It is requested that the Examiner reconsider the Restriction Requirement as to the Group IV-V inventions because these inventions are linked to Groups I-III as process and product by process. It is believed that only a single search is necessary to identify methods for recombinant mammalian cells culture making use of butyrate analogues to induce protein precursor (or zymogen) processing, which represents the disclosure underlying the embodiments as stated in the present Application at page 5, lines 3-9, and therefore to produce mature recombinant proteins such as the two chain-urokinase (tc-uPA). For these reasons, it is requested that an action on all claims be issued.

In paragraph 4 of the Office Action, the Examiner noted that the date was not provided for references cited as CA and CB in the previously submitted Information Disclosure Statement. A supplemental Information Disclosure Statement is annexed to this Amendment with the correct citations to references CA and Cb. Any inconvenience is regretted.

In paragraph 5 of the Office action, the Examiner requested that the specification be amended to add sequence identification numbers at pages 13 and 21.

The sequence identification number for the oligonucleotides provided in the Sequence Listing as Seq ID No 1 and 2 have been added at page 13. No sequence listing was provided for the analysis of the N- and C-terminal aminoacids provided at page 21 and therefore there is no reference number to be added. The reason for not providing any sequence listing was that the sequences provided do not identify any particular peptide, but represent only the analytical results of the Edman degradation. For these reasons, it is believed that the specification is now in conformance with 37 CFR§1.821(a)(1) and (a)(2).

Reconsideration of the requirement to amend the Title is requested. The applicants are concerned that the proposed new Title may convey the impression that the invention is only directed to the production of a single distinct molecule. It is requested that the Examiner consider approving a Title which recites the substance of the preamble of new claim 69.

In paragraph 7 of the Office Action, claims 1 and 40 were

objected to with regard to the use of "an eukaryotic" and "a eukaryotic" in claims 1 and 40. Claims 1 and 40 have been canceled and this ground of rejection has been rendered moot because the newly presented claims use the term "a eukaryotic".

Claims 41, 44 and 46 and the claims that are dependent therefrom were objected to because of the use of the term "chosen among". All of the newly presented claims utilize the expression --selected from the group consisting of--, where appropriate. For these reason, it is requested that this ground of objection not be applied to the newly presented claims.

In paragraph 9 of the Office Action, claims 43 and 44 were objected to because of a number of informalities. The newly presented claims avoid the use of the expressions that were noted by the Examiner.

In paragraph 10 of the Office Action, the Examiner objected to the recitation "tc-UPA" in claims 42 and 43 and to the expressions "HMW" and "LMW" in claim 43.

The acronyms "tc-uPA", "HMW" and "LMW" have been replaced by the full expressions for these abbreviations which are well known in the art. For this reason, it is requested that this ground of objection be withdrawn.

In paragraph 11 of the Office Action, the Examiner stated that claims 1 and 40 were considered to substantial duplicates. These claims have been canceled and the newly presented claims have been drafted to avoid any basis for asserting this ground of objection.

In paragraph 12 of the Office Action, claims 1, 40, 41 and 42 were rejected under 35 U.S.C.§112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter that the applicant regards as the invention.

Reconsideration is requested.

Claims 1 and 40 have been canceled and claim 40 has been replaced with new claim 69. The limitations of former claims 41 and 44 have been combined into new claim 69. New claim 70 corresponds to former claim 42 wherein the term preproenzyme has been replaced when referred to prourokinase with the term

precursor, as suggested by the Examiner. New claim 71 corresponds to former claim 43 and is dependent upon claim 70 and, in addition steps d) through e) have been incorporated into step c) as part of the ion-exchange chromatography as suggested by the Examiner. As noted supra, the acronyms "tc-uPA", "HMW" and "LMW" have been replaced with the full text for each of these terms. New claim 72 corresponds to claim 45 wherein the phrase "are in concentration comprised between 0.1 mM and 20 mM" has been substituted with the phrase "wherein the concentration of said alkanoic acids is in the range from 0.1 to 20 mM". New claim 73 corresponds to former claim 46 and new claim 74 corresponds to former claim 47. Claim 75 corresponds to former claim 48 wherein the phrase "wherein said temperature of incubation in step a) is comprised between 30°C and 37°C" has been substituted with the following: "wherein in step a) said temperature is in the range from 30°C to 37°C". New claim 76 corresponds to former claim 49 wherein the phrase "….wherein said temperature is "comprised between 33°C and 35°C" has been replaced with the " said temperature is in the range from 33°C to 35°C."and new claim 77 corresponds to former claim 50 wherein the phrase "said time is comprised between 48 and 200 hours" has been replaced with "said time is in the range from 48 to 200 hours". New claim 78 corresponds to former claim 51 wherein the phrase "said time is comprised between 72 and 150 hours" has been replaced with "said time is in the range from 72 to 150 hours" and new claim 79 corresponds to former claim 52. Claim 80 corresponds to former claim 53 wherein the phrase "pH values comprised between 5 and 5.8" has been replaced with "pH values in the range from 5 and 5.8".

In claim 69 (formerly 40) and dependent claims the term "mature", which is in the context of this claim referred to proteins, has the meaning to define the final primary structure of a polypeptide, where with "final structure" is meant a biologically active form. The meaning of the term "mature" is exemplified by the protein precursors cited in the Application (p. 4, l. 18-23) where trypsin is the mature form of trypsinogen, chymotrypsin of chymotrypsinogen, hepatocyte growth factor of

proepatocyte growth factor, insulin of preproinsulin, collagenase of procollagenase, etc.

The definition of "mature" according to the Applicant fully overlaps with the one taken from the encyclopedic dictionary: Mc Graw-Hill Dictionary of Scientific and Technical terms" 2nd Ital ed. Zanichelli, 1998. "Mature: Having attained the final state of processing". Even though this definition is referred to aliments it can be appreciated that it fully fits the meaning used in the present Application.

In addition, it is believed that the term "mature" is commonly used in Biotechnology and when referred to proteins, as in the present case, has the meaning of defining the final state attained by a protein after one or more intra- or extra-proteolytic cleavages, such as the removal of the signal peptide or the removal of the pre- or prepro- sequences.

The common use of the term "mature" is confirmed by its use in the Genbank International data-base to indicate the processed form of a protein._

Further use of the term "mature", when referring in particular to proteinases (a category to which also uPA belongs) that are displayed on the Internet in a document (http://www.biology.leeds.ac.uk/nem/science/science2.htm) published by the University of Leeds, which is also enclosed for the Examiner's convenience. The term "mature" is commonly used to refer to the active part of the pre-proenzyme wherein not only the signal (pre-) but also the pro-peptides are cleaved.

For this reason, the term "mature" is used to refer to the secreted and biologically active form of a protein, obtained by proteolytic intra- or extra-cellular cleavage from a precursor.

In new claim 69 the phrase: "factors belonging to the cascade of the complement system", that was used in canceled claim 40, has been deleted. In claim 70 the term pre-proenzyme (formerly in claim 42) has been replaced with the term protein precursor. It is not clear, in new claim 71 (formerly claim 43)a) and the dependent claims, the term "human pre-prourokinase" has not been used. The Applicant wishes to point out that a process for the production of tc-uPA HMW and LMW from cells genetically

manipulated or transfected cells with the cDNA encoding the pre-prourokinase was recited in step a) of former claim 12. Basis for this limitation can also be found in the description, page 4, lines 13-14.

In new claim 71 (formerly claim 43), steps c)-e) the Applicant wishes to point out that steps d) and e) represent the differential elution steps of the ion exchange chromatography defined in step c). Accordingly, step c) in claim 71 has been amended to incorporate steps d) and e).

New claims 71, 72, 75-78 and 80 have been modified to overcome the Examiner's objections to the canceled claims. Each of the bases for the rejection of the canceled claims has been addressed in the newly presented claims and favorable consideration of these claims is requested.

In paragraph 13 of the Office Action, claims 1, 40 and 41 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that at the time the application was filed, the inventor had possession of the claimed invention.

Reconsideration is requested.

New claim 69, which is based on canceled claim 40 has been now limited to three species of enzyme precursor: pre-proenzymes, zymogens and matrix-metallo proteases. The **enzyme precursor** represents the **genus** and pre-proenzymes, zymogens and matrix-metallo proteases represent the **species** belonging to said genus. The Applicant has disclosed the production of an active enzyme (tc-uPA) from a pre-proenzyme (pre-prourokinase) through sc-uPA (prourokinase) which is a zymogen and this disclosure is fully commensurate with the scope of new claim 69.

The pre-proenzymes, zymogens and matrix-metallo proteases are, without any doubt, species falling within the genus of enzyme precursors. With regard to the particular enzyme precursors such as trypsinogen, chymotrypsinogen, plasminogen etc. specified in the Application on p 4, l. 17-23, it should be pointed out that all of them have been fully characterized as to the aminoacid and nucleotide sequences (see for example human

chymotrypsinogen, GenBank Acc. N° M24400, plasminogen GenBank Acc. N° X05199) which is well known and publicly available so that anyone skilled in the art can prepare these materials.See also for example Tomita et al. Biochem. Biophys. Res. Comm., 1989, 158(2), 569-575; Forsgren, M et al. FEBS Lett. 1987, 213: 254-260).

The Applicant may rely on this knowledge in the prior art as a part of the disclosure of the new process which allows for the production of known enzyme precursors in their mature (i.e. active) form. A step of final proteolytic activation is the common feature to the genus described. In addition, the three different precursors now in claim 69, belong to the same family of enzyme precursor. For these reasons, it is requested that this ground of rejection be withdrawn.

In paragraph 14 of the Office Action, claims 1 and 40-55 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that at the time the application was filed, the inventor had possession of the claimed invention.

Reconsideration is requested.

The claims of the present application have been deleted limited to the use of a precursor protein selected from the group of: preproenzymes, zymogens and matrix metallo-proteases. Production of recombinant proteins is a routine method. These methods are described in "Molecular Cloning" Sambrook and Maniatis CSH ed. 1989, and in "Animal Cell Culture" Master JRW ed., 2000). Once the encoding nucleotide sequence is known (obtainable by a simple query with the protein name in GenBank), a skilled worker in the art by using the common general knowledge would easily be able to achieve expression of recombinant proteins belonging to the genus of "enzyme precursors".

The events underlying the production of recombinant mature proteins according to the invention are not merely a peculiarity of the particular protein precursor expressed. On the contrary, the underlying common mechanism relies on the whole expression system comprising the host cell and the culture conditions which

allow for the expression of endogenous proteases.
Generalization is allowed since all the precursors in the claimed genus are activated by proteolytic cleavage (see specification p 4, l. 15-18).

It is also known that processing of several protein precursors either by specific proteases in vivo or by different proteolytic enzymes in vitro, will maintain the same site specificity. For example, three different enzymes: plasmin, kallikrein and trypsin preferentially hydrolyze the peptide bond after a basic aminoacid residue, which allows the peptide bond between Lys and Ile to be hydrolyzed, even though plasmin protease mainly performs in vivo urokinase processing.

As a matter of fact, tc-uPA can be obtained from its inactive precursor sc-uPA either by plasmin in vivo or by kallikrein proteolytic cleavage in vitro (see Merck Index, 11th ed).

Cells used for recombinant protein expression (i.e.CHO, BHK, hybridoma cells) are not just empty translational boxes, but are capable of expressing their sets of endogenous product among which proteases or protease inhibitors, which may eventually interact with the recombinant product altering, modifying it, or as is probably the case here, processing it.

The expression of endogenous proteases in cells used as cell factories is well reviewed for example in Kratje et al.: "Evaluation of the proteolytic potential of in vitro-cultivated hybridoma and recombinant mammalian cells" J. Biotechnol. 1994, 32(2):107-25 (abstract enclosed).

Although clarification of the underlying basic mechanisms was not provided in the present Application, since very burdensome for a product oriented company, the final technical achievement represented by more than a 95% conversion of an inactive urokinase form (sc-uPA), has been shown without any doubt. In figure 1 is compared the same purification performed on the supernatant of cells which have been cultured without butyrate (lane 1) or in the presence of 1.2 mM butyrate (lane 2), where it may be easily appreciated that the sc-uPA (precursor) band is no more present after treatment with butyrate. A

declaration is in preparation on this issue.

As shown, the precursor processing is specifically obtained only after addition of butyrate analogues to the culture medium but not in their absence. Even though this has been experimentally demonstrated in a particular embodiment, the method has a general applicability and has been as such claimed.

The present application contains a working example and in addition, since methods for cloning and expressing protein precursors such as those listed in the application in mammalian cells are well known in the art, the applicant believes that the disclosure is enabling for the entire scope of protection of new claim 69.

With regard to the scope of the claims not commensurate with the enablement provided with particular reference to the extremely large number of alkanoic acids:

- it should be noted that claim 69, formerly 40, has been further limited to specific alkanoic acids and in particular to butyrate analogues comprising butyric acid, sodium butyrate, sodium propionate, magnesium butyrate, tributyrin and phenyl-butyrate and their derivatives or salts thereof.

With regard to the lack of enablement for the kind of ion-exchanger to be used for urokinase purification:

it is common general knowledge that once the isoelectric point of a protein is known, the choice of the ion-exchanger (cationic or anionic) is automatic. Ready to use tables such as table 5.4 on p. 114 of the manual "Protein Purification" by Scopes R.K., Springer Verlag ed., 2nd ed. (3rd encl.) 1987, indicate that an isoelectric point = 7 requires a cationic ion exchanger.

Since the isoelectric point (pI) of urokinase has been known since at least the 1981 publication date of the Miwa's paper (cited by the Examiner) and is in the range from 7.5 to 9.7, (see also the 2000-2001 Calbiochem Catalogue) the skilled person faced with the problem of purifying urokinase would choose a cation-exchanger chromatography.

The specific mention of a <u>cationic</u> exchanger when the pI of a protein is known, as in the case of the particular embodiment

claimed in new claim 71 is deemed to be unnecessary by the Applicant.

In paragraph 15 of the Office Action, claim 1 and 40-42 were rejected under 35 U.S.C.§102(b) as being anticipated by Okabayashi.

Reconsideration is requested.

The method reported by Okabayashi et al. describes the use of butyrate on CHO cultures to obtain an enhancement in the recombinant protein production. An explanation to the effect of butyrate is looked for at the trascriptional level: "Thus butyrate probably exerts its effect by acting at the transcriptional and /or translational level." (see page 584, end of the paragraph before Discussion).

Okabayashi does not mention or suggest whether the urokinase produced is in its active (mature) or inactive form. Moreover, the detection assay he uses to measure the total urokinase produced, does not allow the detection of **active** urokinase but only of the total amount of recombinant urokinase. In other words, the assay does not discriminate between the catalytically active form of urokinase (tc-uPA) and the proenzyme sc-uPA which is catalytically inactive.

The assay is described by the authors on page 582, 1st line as follows:

...This assay involves the use of an agar plate containing **plasminogen** and fibrin; when a sample containing a plasminogen activator is added to a well in the agar, the plasminogen is converted to the active enzyme plasmin.

The Examiner's attention is directed to the fact that an activator of urokinase, plasminogen (converted to plasmin by even small amounts of activated Urokinase) is added into the assay mixture. Since activated plasmin converts pro-uPA (sc-uPA) into active uPA (tc-uPA), in the Okabayashi assay there are no tools for discriminating between the activation of pro-Urokinase because of the butyrate effect, and the activation because of the presence of plasmin in the assay mixture.

In other words, Okabayashi deliberately chose this assay in order to quantify the **total** amount of Urokinase (active and

inactive) without discriminating between the active and the inactive molecular form of u-PA, because conversion from pro-uPA to tc-uPA is intrinsic in the chosen assay.

The Applicant has shown on the contrary that butyrate analogues induce a completely different effect on recombinant mammalian cell cultures: they act as **processing enhancers** favoring or allowing the maturation of recombinant precursor proteins, as disclosed in the application at page 5, lines 3-9.

In summary, by the use of this assay, Okabayashi simply discloses that the addition of butyrate increases the **overall** urokinase production in CHO cells. This is confirmed by the Okabayashi at page 579, lines 10-11 of the Abstract): "The response of cells to butyrate was rapid: a significant increase in urokinase production was observed 6 hours after exposure to 5 mM butyrate".

Contrary to this teaching, the Applicants have disclosed that the assay used for quantifying active Urokinase in the present application was a chromogenic assay (Pefachrome, page 8, lines 15-17), which is completely different from the one used by Okabayashi and which is **exclusively** dependent on the amount of **active** Urokinase (tc-uPA) in the cell culture medium. This data was also confirmed in the SDS-PAGE performed on urokinase purified from the butyrate-treated CHO supernatant, which was demonstrated to be in its active, mature form.

The ratio of active (mature)/inactive Urokinase in culture containing alkanoic acids in serum and protein-free medium has been quantified in the level of at least 95% of the total Urokinase present in the exhausted medium.

According to the experimental data set forth ion the present application, the **addition of butyrate analogues**, to serum- and protein-free media allows for the very efficient **conversion of a precursor protein** such as prourokinase **into the active or mature form of the protein** (tc-uPA). As noted above, **this occurs without adding any external proteolytic enzyme** such as plasminogen to the assay or to the cell culture. For these reason, the Okabayashi reference does not anticipate the newly presented claims.

In paragraph 16 of the Office Action, claims 43-49 and 53-55 were rejected under 35 U.SD.C.§103(a) as being unpatentable over Okabayashi in view of Nobuhara and Miwa.

Reconsideration is requested.

Okabayashi has been distinguished from the claimed invention supra. As a matter of fact no teaching is provided by Okabayashi regarding the fact that the cell culture supernatant contains the enzymatically active tc-uPA molecular species as a consequence of the addition of butyrate. Without a specific indication of the ratio of active/inactive urokinase (information which is completely missing in Okabayashi) the skilled worker in the art would have taken the increase in uPA expression observed by Okabayashi and by combining it with the teaching of Hu et al. which refers to a method for producing recombinant uPA in CHO cells wherein 90% is (inactive) scu-PA, would have only derived that the addition of butyrate analogues increases the production of a product **which is 90% immature or inactive** (Abstract, 3rd l. from the end). On the contrary the teaching of Okabayashi seems to correlate the addition of butyrate to an effect on SV40 promoter, as said in the Discussion and in particular on page 585, lines 1-3: "Thus it appears that recombinant CHO-K1 cells expressing SV40 early promoter-directed genes may offer a convenient model for the study of the effects of butyrate on gene expression". See also at the end of the page: "In these experiments, our goal was to determine if butyrate had any effect on the expression of a specific gene".

Therefore the applicant respectfully submits that Okabayashi fails to render the claimed subject matter obvious. The Nobuhara reference merely discloses a comparison of high molecular weight urokinase and high molecular weight urokinase.

The effect observed by the applicant is different and totally unexpected with reference to the teaching of Okabayashi when combined to the teaching of the secondary references, as far as, as stated in the specification at page 9, lines 1-4 where it was noted that **the addition of butyrate allows for the conversion of urokinase precursors into tc-uPA to an extent of more than 95%.** The production of an active or mature form of a recombinant

protein represents a problem when the cloning of a full precursor is necessary to achieve good or correct expression, and a final proteolytic activation is needed. A step of final proteolytic activation is the property common to the genus of known proteins described.

Production of precursor proteins in their active form is not a major technical problem for proteins purified from physiologic fluids since proteins that are being circulated have undergone all the physiologic cleavages required for their activation in vivo. However it may represent a major problem for recombinant proteins in which the physiological activation is missing.

As far as new claim 71 (formerly claim 43), which has been made dependent on claim 70 and regarding the urokinase embodiment, the applicant has disclosed how to purify in a **single chromatographic step** both the catalytically active tc-uPA isoforms HMW and LMW which are two physically different molecules present in a cell supernatant at very low concentrations.

On the contrary, the ion-exchange chromatography described in Miwa is the third step of a purification process comprising the steps of: 1) concentrating urinary extracts by NH_4 precipitation, 2) gel-filtrating such precipitated material (see Miwa et al., 1981, Results and Discussion page 465, para. Gel filtration chromatography and page 466, para. Ion-exchange Column Chromatograpy) and 3) separating the LMW and HMW urokinase forms. In Miwa, the separation of the LMW and the HMW forms is not achieved in a single chromatographic step (see new claim 71). Moreover, the urokinase concentration is very different in the starting material of the present Application with respect to the one used by Miwa. In Miwa's urinary extracts the concentration is about 3000-fold higher (see page 464, Results and Discussion, 1st par, lines 1-2) than in the CHO supernatant of the present specification (about 0.05-0.1 mg/ml). The skilled worker in the art, faced with the need to purify uPA from recombinant supernatants in very low concentrations as compared to concentrated material, would be directed away from the teaching of Miwa, because the starting materials are at a very different concentration.

An advantage of the claimed present production and purification method (as claimed in new claims 71 and the claims dependent therefrom) is that two active products are separated in a single ion-exchange chromatographic step and that this separation allows one to obtain both products endowed with a high specific activity. In other words the applicant describes a method for making and purifying two physically different products with a single cell culture which allows the same apparatus to be used for two different molecules. This could be in no way derived by the combination of any of the cited prior art references. Since both HMW and LMW u-PA forms are commercially relevant, the applicants believe that in the present application a new and industrially applicable way for obtaining both recombinant products in a purified form from the supernatant fermentation of a single cell culture has been disclosed. For these reasons, it is requested that this ground of rejection be withdrawn.

In paragraph 17 of the Office Action, claims 50-52 were rejected under 35 U.S.C.§103(a) as being unpatentable over Okabayashi in view of Nobuhara, Miwa and Hu.

Reconsideration is requested.

The Examiner has cited a publication by Hu (2000), as an example of production of urokinase in serum-free media. However this publication explicitly mentions that 90% of the product is sc-uPA, the inactive form of urokinase (see Abstract, 3rd line from the end).

On the contrary the present application discloses that in the particular embodiment represented by urokinase, the product is mainly (>95%) in the active tc-uPA form (see page 9, lines 1-4).

Since there is no basis on which to infer from Okabayashi that active tc-uPA (i.e. the mature form of this protein precursor) can be obtained through the use of Na-butyrate, it is not obvious to someone skilled in the art to combine the references cited by the Examiner in order to obtain active products such as tc-uPA by the use of butyrate analogues such as Na-butyrate. For these reasons, it requested that this ground of rejection be withdrawn.

An early and favorable action is earnestly solicited.

Respectfully Submitted

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on 12/8/92

Marked Up Copy of Amendments To Specification:

IN THE SPECIFICATION

Rewrite the paragraph that begins at page 13, line 8 as follows:

--The mixture of cDNA molecules was specifically amplified by PCR with the following 5' and 3' primers:
Oligo 1(5'): $^{5'}$TAGCGCCGGTACCTCGCCACCATGAGA$^{3'}$ SEQ ID NO 1
Oligo 2(3'): $^{5'}$TGGAGATGACTCTAGAGCAAAATGACAACCA$^{3'}$ SEQ ID NO 2--